BIODEGRADATION OF THE OCTACHLOROCYCLOPENTADINE CHLORDANE, BY A MIXED MICROBIAL MAT

A THESIS

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

BY

R. REYNOLD MURRAY

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

JANUARY 1993

ABSTRACT

BIOLOGY

MURRAY, REYNOLD B.S., MOREHOUSE COLLEGE, 1989

BIODEGRADATION OF THE OCTACHLOROCYCLOPENTADINE CHLORDANE, BY A MIXED MICROBIAL MAT

Advisor: Dr. Judith Bender

Thesis dated January, 1993

A chlordane-tolerant strain of cyanobacteria was developed. The cyanobacteria was immobilized on ensiled grass clippings which provided nutrients and added a number of microaerophilic bacteria to the system. Subcultures of this Silage Microbial Mat were treated with 200 mg/l of chlordane. Significant levels of chlordane disappearance were observed in five days. Ensiling bacteria, *Chromatia*, and *Oscillatoria* isolated from this system also demonstrated the ability to degrade chlordane. HPLC, GC/MS and an invertebrate bioassay confirms that the process was biodegradation and not bioaccumulation. This research thus provides an archetype for much needed remediation technology.

ABSTRACT

BIOLOGY

MURRAY, REYNOLD B.S., MOREHOUSE COLLEGE, 1989

BIODEGRADATION OF THE OCTACHLOROCYCLOPENTADINE CHLORDANE, BY A MIXED MICROBIAL MAT

Advisor: Dr. Judith Bender

Thesis dated January, 1993

A chlordane-tolerant strain of cyanobacteria was developed. The cyanobacteria was immobilized on ensiled grass clippings which provided nutrients and added a number of microaerophilic bacteria to the system. Subcultures of this Silage Microbial Mat were treated with 200 mg/l of chlordane. Significant levels of chlordane disappearance were observed in five days. Ensiling bacteria, *Chromatia*, and *Oscillatoria* isolated from this system also demonstrated the ability to degrade chlordane. HPLC, GC/MS and an invertebrate bioassay confirms that the process was biodegradation and not bioaccumulation. This research thus provides an archetype for much needed remediation technology.

NOTICE TO BORROWERS

All dissertations and theses deposited in the Robert W. Woodruff Library must be used only in accordance with the stipulation prescribed by the author in the preceding statement.

The author of this thesis/dissertation is:

Name: R. Reynold Murray

Street Address: 2157 Lenox Road N.E.

City, State and Zip: Atlanta, Georgia 30324

The directors of this thesis/dissertation are:

Professor: Dr. Judith Bender

Department: Biological Sciences

School: Arts and Sciences, Clark Atlanta University

Office Telephone: (404)880-8809

Users of this thesis/dissertation not regularly enrolled as students of the Atlanta University Center are required to attest acceptance of the preceding stipulation by signing below. Libraries borrowing this thesis/dissertation for use of patrons are required to see that each user records here the information requested.

NAME OF USER	ADDRESS	DATE	TYPE OF USE

ACKNOWLEDGEMENTS

The ban on chlordane use and distribution is rigorously enforced in the United States, the country where it is made. This work is therefore indebted to Mr. J. Young of Batelle Laboratories and Dr. J. Gould of Georgia Institute of Technology for providing the samples that made the work possible. Dr. Gould's insightful comments were also useful in the development of extraction and analytical procedures.

My gratitude and respect are extended to Dr. J. Bender, my supervisor. Her numerous searching questions and tangential suggestions enabled me to see beyond my own narrow plans. I am also grateful to Dr. P. Phillips for his editorial eyes and general resourcefulness, Dr. J. Stewart for her multifaceted ideas, and Dr. W. Dashek for technical advice.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

LIST OF ABBREVIATIONS

GC/MS .... Gas Chromatography/Mass Spectroscopy

HPLC ..... High Performance Liquid Chromatography

SMM ..... Silage Microbial Mat

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

CHAPTER I

INTRODUCTION

Imagine ... A world with no flowers
A world with no bees.
A world with no birds
To sing in the trees.

Because they do not always fit into our scheme of things, insects, birds, plants and animals are often labelled pests. Many insects are responsible for the transmission of fatal diseases like malaria, trypanosoma, and elephantiasis to name a few. Others cause billions of dollars worth of damage to food crops each year (Pimentel et al., 1991). To control these "undesired" organisms, pesticides were developed.

The term pesticide embraces a wide range of toxic chemicals used in the control and attempted eradication of "undesired" life forms. Among these chemicals are insecticides (compounds designed to control insects) and herbicides (compounds designed to control small plants). These two compounds have been sighted as major environmental contaminants (Black and Koeman, 1984). Of the 435 million kilograms of pesticides used annually in the United States, 69% are herbicides and 19% insecticides (Pimentel et al., 1991). The Windward Islands (four islands in the South Eastern Caribbean), with a total area of approximately 800

square miles, used over 1.8 million kilograms of pesticides in 1988 (Vincentian News, 1990). Per unit area, the Windward Islands used approximately twenty (20) times the amount of pesticides used in the United States. Ironically, the share of crop yields lost to insects has nearly doubled during the last 40 years despite the more than ten-fold increase in both the toxicity and amount of synthetic insecticides used (Pimentel et al., 1991). The intent and manner of use of pesticides lends credence to the idea that man can become exempted from the biological laws which governs his life and the lives of perhaps two million species of plants and animals that share the planet. Man has thus been termed ecologically dominant (Rudd, 1966). This dominance has left us with a slew of contaminants and very little knowledge of how to rid ourselves of their taunting presence. The objective of this research was to address this problem by developing a pesticide degradation system with field application potential.

CHAPTER II

LITERATURE REVIEW

THE DILEMMA

In a universe where humans reign supreme, our selfish motives are directed towards our own comfort and convenience with little regard for the rest of the ecosystem upon which our lives depend totally. The use of pesticides is widespread and indiscriminate. We have little enough understanding of the dynamics of the biological environment, yet we are altering them at a rate that precludes our understanding (Rudd, 1966). When confronted with disappearing species and contaminated food, we justify a special treatment as instigators of a practice that both benefits and harms us.

Of course, it would be difficult to eliminate the use of pesticides without appropriate and effective substitutes. Pesticide use may be warranted at least temporarily in cases where disease vectors must be controlled or where climatic conditions make it impossible to farm without the use of selected pesticides. Over the last decade however, chemical industries have come to life at an alarming rate, their commercial and waste products are randomly spread across the environment. Lack of knowledge regarding the fate of these chemicals in major aquatic systems has resulted in confusion

and poorly defined issues regarding the hazards of chemical contamination and loss of environmental quality (American Chemical Society, 1972).

The hazards of pesticide use are not restricted to the environment. Users often ignore safety standards and put themselves at enormous risk. In Australia, heptachlor is sometimes applied by bare-chested applicators wearing sandals (Stacey and Tatum, 1985). In Argentina, untrained persons with no protective clothing can apply pesticides to a wide range of food crops (Camara de Sanidad Agropecuaria y Fertilizantes, 1985). This scenario is common in several countries around the world especially the so called "Third World Countries". Worse still is the fact that the use of many of these pesticides is banned in the countries where they are manufactured (Greenpeace Report, 1989). The paradox is that these pesticides are used in fields where export crops are grown. Rudd (1966) points out that except for deep rooted trees and shrubs, the entire plant structure in a treated area, from root tip to meristem, falls within the contamination zone.

RATIONALE FOR PESTICIDE SELECTION

This study was undertaken to help the island nation of St.Vincent address its pesticide problems. St. Vincent is a small volcanic island in the Eastern Caribbean. Its fertile volcanic soil gives the island its only natural resource

volcanic soil gives the island its only natural resource which nurtures a rich tropical ecology. As rich as the ecology might be, it is very fragile because of the size of the island (150 sq. miles) and its relative youth in geographic terms.

Despite the petite nature of the island, approximately thirty (30) different pesticides are listed as having been used during the last twenty years (St. Vincent and the Grenadines Agriculture Report, 1989). Of these pesticides, paraquat and glyphosphate are the most widely used. These pesticides are deactivated almost immediately after contact with soil because of tight adsorption to soil particles (Hansen, 1983). Paraquat adsorption to soil particles is irreversible thus it is used with relative safety to kill weeds (Kennedy et al., 1990). However, this does not take into account the possible effects on the soil's micro-flora and fauna. Little is known of such effects. Comparatively few invertebrate impact studies have been carried out with paraquat but no adverse effects on macroinvertebrates were noted when experimental treatment was done in lakes in the United Kingdom (Muirhead-Thomas, 1989).

The next group of pesticides used on the island are the organochlorines; chlordane, chloramben and chlorobromuron. Chlordane (IUPAC name 1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,-7a-hexahydro-4,7-methano-1H-indene) was the focus of this study. Chlordane is an organochlorine

and 100 compounds (Parlar, 1979; New York Department of Environmental Conservation, 1986). Velsicol Chemical Corporation is the world's sole producer of chlordane and heptachlor (EPA, 1986a), two of the most hazardous and persistent pesticides on the international market. Heptachlor contains 21% gamma-chlordane. Chlordane, available since 1947, was banned by the U.S District Court of the District of Columbia in 1988. The decision to ban the compound followed a series of shocking discoveries and ten years of legal debating (Baxter, 1988). Between 1987 and 1989, Velsicol exported approximately 4.8 million pounds of chlordane and heptachlor to 25 countries, some of which re-exported to other countries (Greenpeace Report, 1989). The flow of poison came full circle in 1988 when chlordane concentrations in beef imported to the United States from Honduras, were found to be eight times the permitted level. The U.S. Food and Drug Administration has detected heptachlor in a variety of imported fruits, vegetables and cheese, also above allowable levels (Greenpeace Report, 1989). In 1986, feeding of heptachlor-contaminated mash to dairy cows in Arkansas, Missouri and Oklahoma resulted in extensive contamination of milk and other dairy products. Heptachlor levels in milk reached 120 times the federal residue standard and at least 470,000 gallons of heptachlor-contaminated milk had to be destroyed (Weisskopf, 1986). In the United States over 30 million homes have been treated

with these pesticides and many families have been forced to leave these homes after chlordane from termite treatments contaminated their living quarters and were impossible to decontaminate completely (Hearing Records, H.R. 2622, 1987). In 1986, EPA review of chlordane and heptachlor concluded that the products were "probable human carcinogens", tumor promoters, persistent and bioaccumulative in the fatty tissue of wildlife and humans. Both chlordane and heptachlor are easily absorbed through the skin (Gosselin, 1976) and are known to pass through the placenta and to be excreted in breast milk (Stacy and Tatum, 1986; Curley et al., 1970). Chlordane poisoning may result in nervous system disturbances such as headache, disorientation, muscle twitch and lack of coordination (EPA, 1989). Chlordane is reported to have caused malignant liver tumors, increased incidence of breast cancer and soft tissue sarcoma in four strains of mice (Becker and Sell, 1978; U.S. Department of Health, Education and Welfare, 1971). The oral LD50 of chlordane in rats is 343 mg/kg (Merck Manual, 1983). An association was also found between primary brain tumors in children and termicide exposure at home (Epstein and Ozonoff, 1987). Chlordane has been found to be mutagenic in the bacterial Ames test (EPA, 1986b). There is hardly an animal, a body system or a biological process that cannot be adversely affected by exposure to chlordane and/or heptachlor (Sherman, 1988).

AN ENVIRONMENTAL HAZARD

Chlordane does not only exhibit toxicity in animals but, like other organochlorine compounds, it exhibits the characteristics of persistence, bioaccumulation and penetration into food stuffs (Moye, 1980), making it a ubiquitous environmental contaminant. As a consequence of high usage and improper disposal technologies, many soils and subsurface water bodies are seriously affected by chlorinated hydrocarbons (Kearney et al., 1988) often in combination with other organic waste (Fliermans et al., 1988; U.S. Department of Energy, 1989). Chlordane binds tightly to organic material and hence moves very slowly through the soil (Baxter, 1988). This fact, coupled with an estimated half life of four years in soil (EPA, 1975) accounts for the persistence of the compound. Soil sediments and animal tissue from the Yazoo National Wildlife Refuge, Mississippi, were found to contain residues of twelve organochlorine pesticides (Ford and Hill, 1991). Toxicological reports tell of chlordane in atmospheric dust over Mexico (Kearney and Kufman, 1988). Both chlordane and heptachlor are known to be highly toxic to fish, aquatic invertebrates and birds (EPA, 1986a). The Georgia Department of Natural Resources has issued an advisory to fishermen warning of chlordane levels in excess of FDA standards in several species of fish in the Chattahoochee River (Georgia Department of Natural Resources, 1991). The

same is true of several rivers across the United States from California in the West to Washington, DC in the East (Baxter, 1988).

MICROBES IN DEGRADATION

Physical methods of pesticide degradation are impractical, for once the pesticide has been used, it can not be easily pooled. Even if this was possible, the by-products of physical degradation are often more hazardous than the pesticides themselves. Agril (1976) reported the production of large quantities of toxic gases when he tried thermal degradation of chloramben. It is, therefore, practically impossible to discuss the behavior of pesticides in the environment without considering the role of microbes (Matsumura et al., 1972). Because microbes are so versatile in their metabolic capabilities, they can often utilize synthetic molecules such as pesticides, as substrates (Anderson, 1990). Steiert et al. (1987) reported pentachlorophenol (PCP) being degraded by *Flavobacterium*. Degradation of chlorinated herbicides in ground water by a group of naturally occurring bacteria was demonstrated by Cavalier et al. (1987). A microbial biofilm capable of degrading PCP was cultured by Crawford and O'Reilly (1988). The fungi *Rhizoctonia solani*, (Kearney et al., 1988), and *Phanerochaete chrysisporium*, (Bumpus, 1988; Steiert et al., 1987; Kennedy et al., 1990) were also implicated in the

degradation of chlorinated hydrocarbons. Mondecar et al. (1991) reported the degradation of trinitrotoluene (TNT) by a cyanobacterial mat (mixed microbial community dominated by cyanobacteria). Phillips (1991) also use the cyanobacterial mat for the degradation of hydrocarbons (naphthalene, phenanthrene, chrysene and hexadecane). Bacteria are capable of utilizing aromatic hydrocarbons as sources of energy (Rochkind et al., 1990; Barth, 1986). Chlorinated alkenes like trichloroethylene (TCE) are co-metabolized by methanotrophic microorganisms (Wilson and Wilson, 1985).

Remediation of subsurface contaminants may require long-term treatment at considerable cost (Vogel and McCartey, 1985). Mere transfer from a poorly controlled environment to waste storage or atmospheric venting may alleviate immediate concerns without detoxifying the waste (Phelps et al., 1990). However, remediation technologies which mineralize toxicants on site are the most desirable.

Cyanobacteria are prokaryotic organisms with an extensive thylakoid membrane structure running along the inner surface of the plasma membrane. These thylakoids may be the product of invagination of the plasma membrane (Fogg et al., 1973). Whatever the origin, the thylakoids are a rich source of chlorophyll a. Under photoautotrophic conditions, cyanobacteria are capable of extended growth with light energy driving the formation of ATP and NADPH needed for the assimilation of carbon dioxide (Smith, 1982).

As they grow, cyanobacteria secrete large quantities of mucopolysaccharide into their cell walls allowing cells to adhere together in the form of mats. Paerl (1982) has shown that cyanobacteria are frequently the site of intense and varied bacterial and fungal colonization.

NATURAL MATS VS CONSTRUCTED MATS

The cyanobacterial mat developed for this work can be classified as a constructed mat as opposed to a naturally occurring mat. In naturally occurring mats, bacterial and fungal populations are regulated by the availability of the various by-products needed for their metabolism. Such a dynamic, symbiotic ecosystem must be the result of species succession resulting in a stratified mat with several microzones (Paerl and Kellar, 1979). These microzones localize oxidative and reductive processes (Bender et al., 1991) leaving the chemical and physical environment drastically different from ambient conditions. The biology and chemistry of the mat are as varied as the number of layers and microzones. A comprehensive list of such variations compiled by Paerl (1982) includes the following:

I. Photosynthetic generation of organic carbon compounds which can be utilized by heterotrophs.

II. Excretion of growth factors and vitamins by bacteria utilizing algal photosynthate.

III. Nitrogen transformation steps restricted by specific oxygen requirements.

IV. Creation of microzones with varying oxygen levels suitable for specific redox reactions.

V. Respiration producing carbon dioxide which promotes specific metabolic reactions and pH requirements.

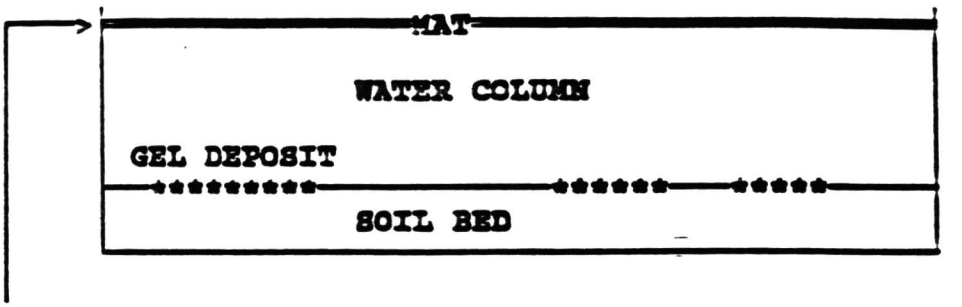
Some of the foregoing processes and reactions are illustrated in Table 1. It is important to note, however, that the occurrence of these reactions is not linear. Take the case of the sulfate produced by the sulfur-oxidizing bacteria such as *Chromatia spp.*; it is converted to hydrogen sulfide during anoxic respiration by sulfur reducing bacteria such as *Disulfovibrio*. The hydrogen for this reaction may be produced by the nitrogenase activity of nitrogen-fixing bacteria (Caumette, 1989). The respiratory by-product, hydrogen sulfide, is a major ingredient of anoxic photosynthesis carried out by *Chromatia spp*. The entire system is intricately tied together by the ability of gases to diffuse, the diurnal changes in light intensity, and the ability of some organisms to tolerate variations in light and oxygen intensity making it possible for them to live in more than one zone.

Table 1. Some interactions within a microbial mat.

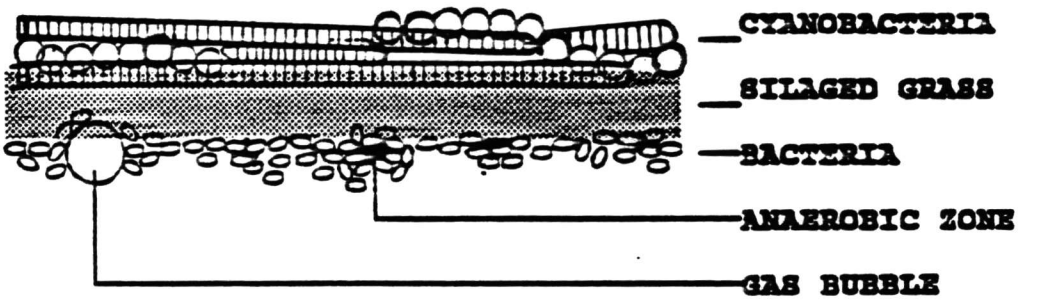
ZONES	SELECTED MICROBES	PROCESSES	CHEMICAL REACTIONS
oxic	cyanobacteria	oxic photosynthesis	$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$
semioxic	purple bacteria	anoxic photosynthesis	$2CO_2 + H_2S \rightarrow 2(CH_2O) + SO_4^-$
anoxic	sulfur reducers	anoxic respiration	$5H_2 + SO_4^- \rightarrow H_2S + 4H_2O$

In the constructed mats, the interdependence of the organisms is the same as in the naturally occurring mats but the number of species and their population sizes are altered to meet specific needs. *Oscillatoria spp.*, the predominant organism in this constructed mat, is a filamentous cyanobacteria covered by a thick sheath of polysaccharide (Fogg et al., 1973) which helps in the gliding movement of mobile species. The growth of this cyanobacteria is stimulated by ensiled grass clippings (Fig.1) which is a ready source of organic substrates like lactic acid and acetic acid as well as ensiling bacteria. The acids are produced during the ensiling process. In the first phase of this process, the hemicellulose of the grass cell wall is broken down and aerobic bacteria convert the available glucose and fructose to formic acid and acetic acid. In the next step, homofermentatives like *Lactobacillas casei* converts glucose to lactic acid and acetic acid while heterofermentatives like *Leuconostos cremoris* converts glucose to ethanol; pentose and fructose to acetic acid (Sale, 1986). In addition to providing nutrition, the ensiled grass serves as fiber to cross link the mat and anchor the microbes. This is especially important as immobilization increases the economic value of the mat by permitting its reuse. Immobilization can confer physical stability, biomass retention, limitation of cell growth and

Fig. 1. A schematic diagram of a silage microbial mat showing stratification and microzones; anaerobic zones, clusters of bacteria and oxygen gas bubbles.



STRUCTURE OF FLOATING SILAGE-MICROBE MAT:



greater ease of multi-enzyme reactions (Kerby and Stewart, 1988). If the silage is applied to a system containing soil, the chemical components of the silage facilitates a chemotactic response from soil microbes (Vatcharapijarn, unpublished data, 1988). These microbes may be nitrogen fixers that support the establishment of phototrophs and symbionts. Among the phototrophs are the purple sulfur bacteria (*Chromatia spp.*) and the brown sulfur reducers (*Heliobacteria*) (Cohen and Rosenberg, 1989). When mat cultures are grown without a soil base, colonies of these various bacteria are often added. This is another variation in constructed mats.

RESEARCH OBJECTIVE

The objective of this study was to provide a pesticide bioremediation technology which has low-cost potential for field application. Given the biological complexity, the reported versatility, its ubiquitous nature and primeval genesis, my hypothesis was that a cyanobacterial mat dominated by *Oscillatoria spp.* would be able to degrade chlordane. Such degradation would have both environmental and biomedical relevance. Not only would the environment be free of the toxic effects of this compound, but its related biomedical problems also.

To this end, a chlordane-tolerant strain of cyanobacteria was developed. The cyanobacteria was then

cultured with ensiled grass clippings and the associated ensiling bacteria to produce a silage microbial mat (SMM). Cultures of SMM were exposed to very high levels of chlordane, samples were harvested daily and analyzed to determine the degradative efficiency of this system.

CHAPTER III

MATERIALS AND METHODS

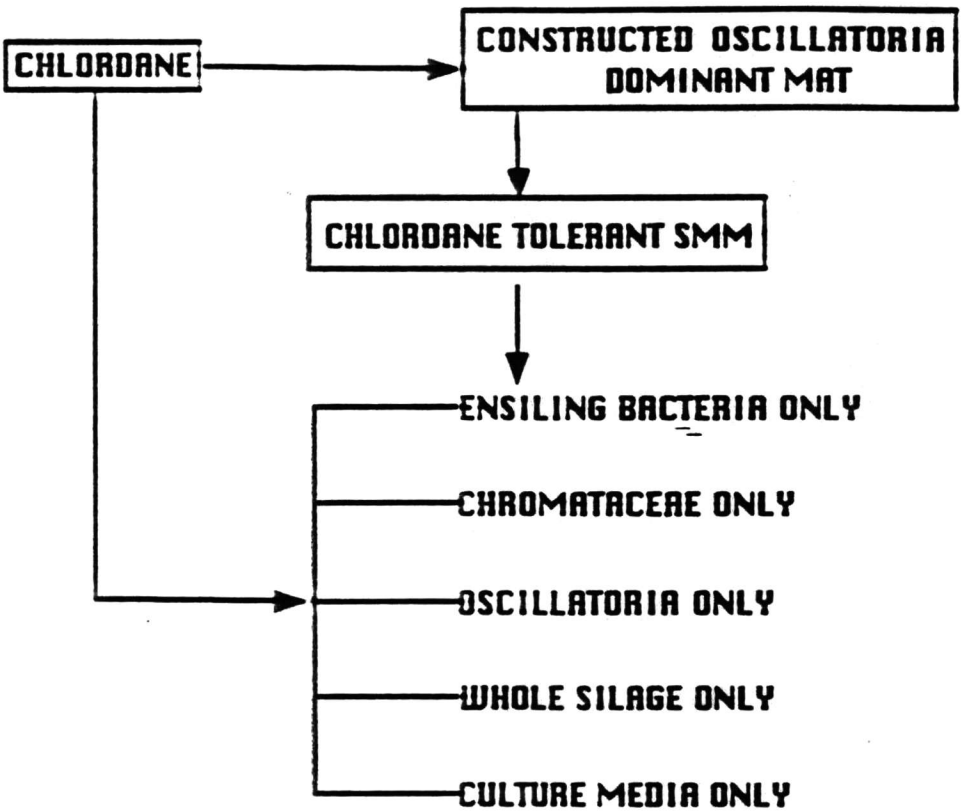
Two categories of experiments are outlined in these methods: (1) Constructed mat experiments; (2) Individual microbial species experiments (Fig. 2).

CONSTRUCTED MAT EXPERIMENTS

MAT DEVELOPMENT

In these experiments, cyanobacterial mats dominated by *Oscillatoria spp.* were used. Cyanobacteria from stocks originally obtained from a rock substrate in Tampa, Florida, were streaked onto agar plates enriched with Allen and Arnon media (Allen and Arnon, 1955) and allowed to develop under sixty watt incandescent lamps generating 20 lux of light as measured by a Pasco photometer, model 8020. The temperature was kept at approximately 25 °C. Five days later, the strains were observed under the light microscope. *Oscillatoria spp.* dominant areas of these plates were subcultured onto new plates. It should be noted that these *Oscillatoria spp.* dominant cultures still carried other associated microbes such as *Chromatia spp.* Cultures from the second round of plates were transferred to flasks containing 50 mL Allen and Arnon media and 2 g of ensiled

Fig. 2. A schematic diagram of the experimental design. Each group represents a separate experiment.


CHLORDANE
CONSTRUCTED OSCILLATORIA DOMINANT MAT
CHLORDANE TOLERANT SMM
ENSILING BACTERIA ONLY
CHROMATACEAE ONLY
OSCILLATORIA ONLY
WHOLE SILAGE ONLY
CULTURE MEDIA ONLY

grass clippings. The ensiled grass added lactic acid, acetic acid and a group of naturally occurring microaerophilic ensiling bacteria to the system. These flasks were placed into a Biotronette Mark III environmental chamber lighted by four twenty-watt fluorescent lamps generating 50 lux of light. The lights were regulated by an Intermatic timer which allowed sixteen hours of light per day. The temperature in the environmental chamber was 28 °C ± 1 °C. A slimy, green silage microbial mat (SMM) developed in about seven to ten days, covering the entire surface of the media and penetrating to a depth of 5 to 10 mm. During the first two days of growth, the pH, measured with a Corning PS-15 pH meter, was 4.5 ± 0.5, with a thin bacterial film covering the surface. On the third day, green patches of cyanobacteria were usually apparent and the pH rose to 9.0 ± 0.5 by the sixth day of culture. Oxygen levels in the system, determined by an Otterbine Sentry III oxygen/temperature monitor, changed from 3 ± 1 mg/L on the first day to 10 ± 2 mg/L on the sixth day. This change in oxygen levels reflected the increase in the cyanobacterial population accompanied by increased photosynthesis.

DEVELOPMENT OF CHLORDANE-TOLERANT MAT

Chlordane was a gift from J. Young of Battelle Pacific Northwest Laboratory, Richland, WA. It contained 74% chlordane, 10.2% petroleum distillate, and 15.8% inert

ingredients. The stock solution contained 0.96 kilogram of chlordane per liter.

The SMM developed from the culture system previously described, was acclimated to chlordane by adding extremely low levels of chlordane to the culture flask (2 to 3 mg/L) and gradually increasing the concentration by 2 mg/L each time fresh media was added to the flasks. After three months of treatment, the cyanobacterial mat reached a tolerant level of approximately 3,000 mg/L. This acclimated mat was then used as inoculum for the new SMM used in the degradation experiments.

DEGRADATION OF CHLORDANE BY SMM

The degradation experiments were performed in 250 mL Pyrex beakers placed into an environmental chamber under conditions described above. To avoid evaporation of the water columns, the beakers were covered with clear plastic cling wrap which was held in place by rubber bands. Each experimental beaker contained 2 g of silage (wet weight), 50 mL of Allen and Arnon media, and approximately 0.5 cm^2 of chlordane-tolerant mat cut into smaller pieces and spread over the water column surface. Triplicate test cultures were prepared in this way. After seven days of growth, 200 mg/L of chlordane were added to each culture and the beakers swirled gently to facilitate dispersal of the chlordane.

Triplicate samples of experimental cultures were analyzed via HPLC one hour after injection of the chlordane, and daily thereafter for five days. Controls lacking mats were similarly treated.

EXPERIMENTS IDENTIFYING THE MICROBES ACTIVE IN CHLORDANE DEGRADATION

It was necessary to determine whether degradation of chlordane was the combined effort of all members comprising the total mat, or alternatively, whether it was the responsibility of a single species of microbe within the mat. To this end, cultures of some individual species comprising the mat were isolated and used in degradation experiments.

TESTING OF MIXED ENSILING BACTERIA POPULATION

To obtain the bacterial consortium from the ensiled grass, a sterile mixture of Allen and Arnon media and soil-wash (1:1) was used. Soil-wash is prepared by mixing top soil and/or estuary soil and water in a 2:1 ratio (v/v). The mixture is allowed to stand for 24 hours before the liquid is separated by filtration. The clear filtrate is autoclaved, cooled, and stored in a refrigerator. Soil-wash thus contains inorganic salts leached from the soil. Ensiled grass clippings was soaked overnight in the aforementioned mixture. The following day, the thin

bacterial film which appeared on the surface of the media was taken up with a transfer pipette and placed in a 250 ml flask containing Lauria broth purchased from Sigma Chemical Company. This flask was sealed with a screw cap. Two days later the dense bacterial population was separated from the remains of the broth by centrifugation in a ICE HN-SII centrifuge at 1,000 xg for 15 minutes. The resulting pellet was washed twice with distilled water. The bacteria were then diluted to 2 x 10^{12} cells per ml. This poulation count was determined from the optical density obtained by spectrophotometry. Fifteen ml of this culture were added to 35 ml of a sterile 4:1 (v/v) mixture of Allen and Arnon media and soil wash. Cultures thus prepared were treated with 200 mg/l of chlordane. Triplicate samples of these cultures were analyzed daily by HPLC.

TESTING OF PURPLE SULFUR BACTERIA

In a complete mat system, *Chromatia spp.* colonize immediately beneath the mat. To obtain colonies of this species, a Pasteur pipette was pushed down the side of a culture tube containing whole mat to a depth of about 5 mm below the mat. Approximately 5 uL of media from this area was taken and dispensed into a 5 ml test tube containing media made by adding equal volumes of the following: Allen and Arnon media, silage/algae extract, and soil wash. Silage/algae extract was prepared by blending 5 g of ensiled

grass clippings and 5 g wet algae in a Presto blender with 100 ml of water. After blending, the mixture was filtered through a Buchner funnel fitted with Whatman No.2 filter paper. DIFCO Bacto-agar was added to this filtrate at 3 g/l and the mixture autoclaved at 150 °C for 10 minutes. When the culture tubes were placed in subdued light (5 lux) at room temperature, they appeared blood red (colored by *Chromatia spp.*) in five days. It should be noted that, in obtaining the *Chromatia spp.*, sterile plating procedures were not used, hence there may have been colonies of heterotrophic bacteria interspersed with the *Chromatia spp.* Experimental tubes containing 5 ml of this culture, 10^9 cells per ml, 15 ml water and 200 mg/l of chlordane were analyzed daily.

TESTING OF *OSCILLATORIA spp.*

Oscillatoria spp. cultures developed from hermatogonia were used for this purpose. Hermatogonia are sporelike fragments which break away from a mature cell. These hermatogonia escape into the atmosphere free of all other microbes. By constructing a hood over a flask containing *Oscillatoria spp.*, the hermatogonia can be channeled into a beaker containing sterile growth media. Once in the growth media, these hermatogonia developed into mature filamentous cyanobacteria population. These cells were then cultured into an *Oscillatoria spp.* mat using sterile ensiled grass in

sterile media and capped with clear plastic cling wrap. The *Oscillatoria spp.* mat differs from SMM in that, except for *Oscillatoria spp.*, it lacks all the other microbes present in SMM. These *Oscillatoria spp.* mats were treated in an identical manner to the SMM.

TESTING SILAGE AND GROWTH MEDIA

To test the degradative ability of the silage, it was necessary to eliminate all associated bacteria. To this end, the ensiled grass was autoclaved at 150 °C for 30 minutes. Two grams of this silage (wet weight) was then put into a beaker with 50 ml of Allen and Arnon media and 200 mg/l of chlordane. Triplicates of this system were analyzed daily for five days.

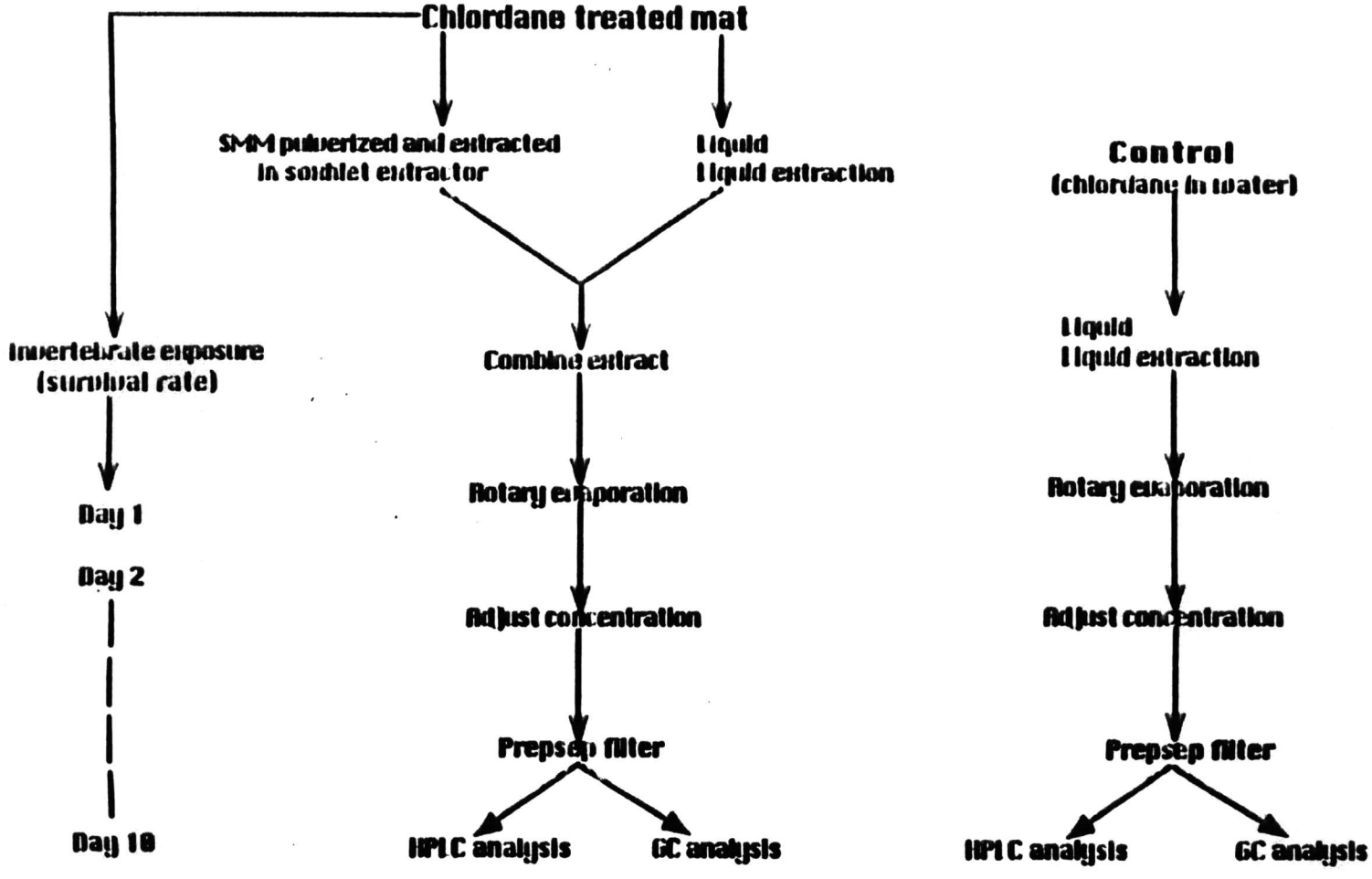
Triplicates of beakers containing 50 ml of growth media treated with 200 mg/l of chlordane were also analyzed daily for five days.

EXTRACTION PROCEDURES

Adapted procedures of Wheeler and Thompson (1980) were used for chlordane sampling, extraction, cleanup and detection (Fig. 3). Each culture was prepared for analysis by extraction with twice its volume of chloroform (an effective solvent for chlordane). The solid portions (SMM) of each system were pulverized then leached with 50 ml of

Fig. 3. Extraction procedures. All samples analyzed followed the order outlined. The invertebrate exposure was used to determine the level of toxicity in the mat.

Fig. 3


Chlordane treated mat
SMM pulverized and extracted in soxhlet extractor
Liquid Liquid extraction
Control (chlordane in water)
Invertebrate exposure (survival rate)
Combine extract
Liquid Liquid extraction
Day 1
Day 2
Day 10
Rotary evaporation
Rotary evaporation
Adjust concentration
Adjust concentration
Prepsep filter
Prepsep filter
HPLC analysis
GC analysis
HPLC analysis
GC analysis

chloroform via a Soxhlet Extractor. Each extraction ran for two hours during which time six to eight cycles of reflux took place. The flask at the bottom of the extractor was heated by a water bath kept at approximately 60 °C. The aqueous portion of each system was washed three times by shaking vigorously for three minutes in a separatory funnel with 20 ml of chloroform. The total extract from the separatory funnel (60 ml) was combined with the extract from the Soxhlet Extractor and rotated in a 125 ml round bottom flask on a Labconco rotary evaporator. After solvent evaporation, the flask was removed from the evaporator and 1 ml of high performance liquid chromatography (HPLC) grade methanol was added. The flask was swirled for five minutes to thoroughly dissolve any chlordane adhering to the glass surface. The resulting solution was then filtered through a 0.45 um Nylon Acrodisc filter at the tip of a syringe. The final solution was collected in a small vial from which 10 ul was analyzed via HPLC.

HPLC ANALYSIS

Although gas chromatography has been the primary method for trace analysis of pesticide residues in environmental samples since the mid-1950s, the principal detection method used in this work was the more recent HPLC; first reported used by Henry et al.(1971).

HPLC sample analysis was performed on a Beckman Instrument driven by Systems Gold software (Kennedy et al., 1990) and equipped with a Whatman reverse-phase RAC II column 5 (ODS-3; length 10 cm, I.D. 4.6 mm). The mobile phase was a mixture of methanol and water run on a 60/40 linear gradient (Moye, 1980). The UV detector was set at 254 nm. A flow rate of 2 ml/min with a total elution time of 20 minutes (Moye, 1980; Kirkland, 1973) completed the method.

GAS CHROMATOGRAPHY/MASS SPECTROSCOPY ANALYSIS

Gas Chromatography/Mass Spectroscopy (GC/MS) was used as a second method of chemical analysis. The clean up and extraction procedures for this method were identical to those for HPLC. After extraction, 10 uL of sample was injected into a Hewlett Packard 5890 Series II GC/MS equipped with a SB5 column, using methanol as the solvent. The oven was programmed to increase from 120 °C to 220 °C in 10 minutes then to 260 °C in 5 minutes. The solvent delay time was 3 minutes. Under these conditions, chlordane has a retention time of 15.5 minutes. All peaks on the chromatogram were identified by comparison with internal standards in the computer's library.

INVERTEBRATE BIOASSAY

In addition to the chemical analytical methods described above, an invertebrate bioassay was used as an index of decreasing chlordane toxicity.

Copepods, class copepoda, are common in aquatic and semi-aquatic habitats. In many fresh water copepods, the copepodid stage or even the adults secrete a cyst-like covering during unfavorable conditions. Locked in this cyst, they become inactive and are able to withstand desiccation (Barnes, 1980). In this way, the organism survives periods of droughts. Occasionally, soil samples containing dormant copepods in various stages of development, are inadvertently used in mat culture systems in the laboratory. These organisms emerge from their cyst and feed avidly on the SMM. Copepods obtained in this way were used in this bioassay.

SMM cultures identical to those used in degradation experiments were used in this case. The systems were treated with chlordane at 200 mg/l. One hour later 15 copepods were put into the system. The animals were observed until all physical signs of life ceased. This routine was followed for twenty days. Changes in copepod survival times were correlated with chlordane degradation data.

STATISTICAL ANALYSIS

In order to compare the rates of degradation of controls to experimental treatments, and to assess the statistical value of such experimental results, a MINITAB data analysis software package was used. All hypotheses were tested at a 95% confidence interval.

CHAPTER IV

RESULTS

Studies on the microbial biodegradation of the chlorinated compound chlordane, were carried out. All extracts from experimental cultures were tested by HPLC for the presence of chlordane. GC/MS analyses were done on selected cultures to verify the HPLC findings. Figure 4a shows an HPLC chromatogram for a chlordane standard with a concentration of 200 mg/l, obtained under conditions described in chapter 2. Under those conditions, chlordane produced multiple peaks consistent with the report of Osselton and Snelling (1986). Figure 4b shows a GC/MS chromatogram obtained when a chlordane standard with a concentration of 600 mg/l was subjected to the conditions described in chapter 2. In the first set of experiments, the consortium of microbes were intermingled in a SMM. The SMM was grown under simulated environmental conditions in an environmental chamber. Each culture was treated with 200 mg/l of chlordane and analyzed separately. All experiments were done in triplicates and run over a period of five days. Figures 5a, 5b, and 5c shows HPLC chromatograms for one, three and five days, respectively, after SMM treatment with chlordane. The decrease in total area of the peaks between 7.5 and 13 minutes is evidence of the decrease in chlordane

Fig. 4a. HPLC chromatogram of chlordane standard. Peaks with retention time between 8 and 13 minutes are the important ones in this chromatogram. The other peaks represent inert ingredients.

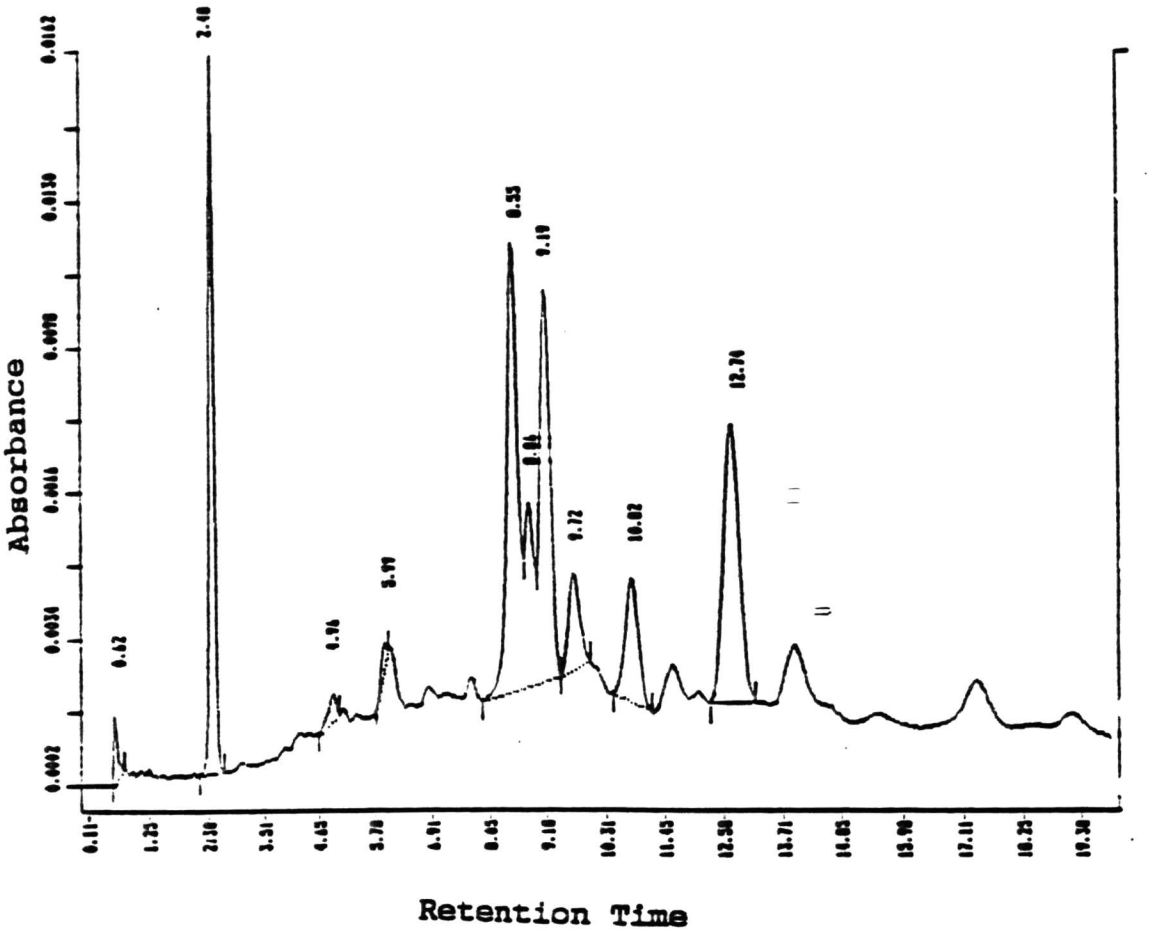

Absorbance
Retention Time

Fig. 4b. GC/MS chromatogram of chlordane standard. Peaks with retention time between 15.5 and 16.5 minutes are the ones considered here. The other peaks represent plasticides and other inert ingredients.

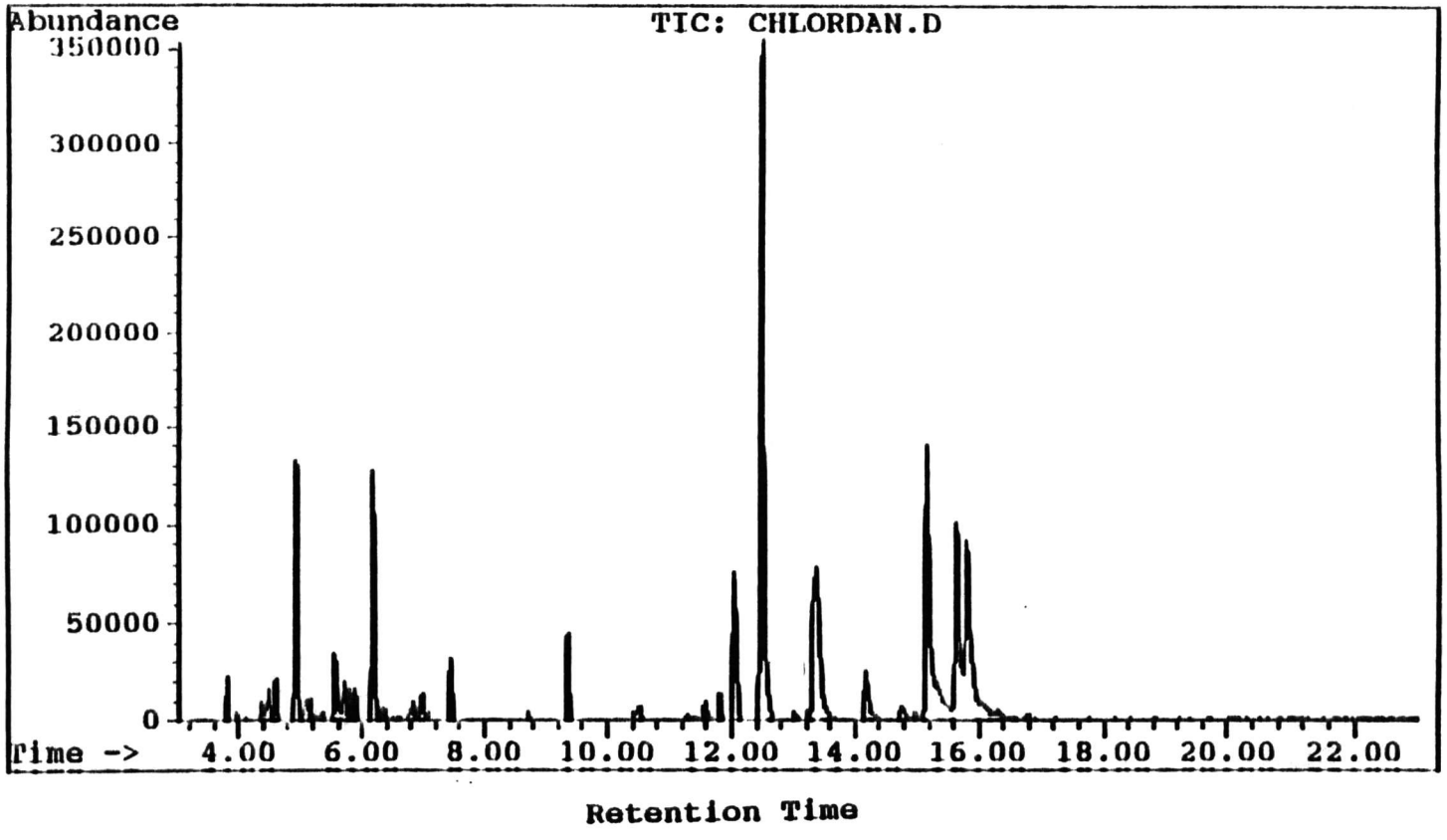

Abundance
TIC: CHLORDAN.D
350000
300000
250000
200000
150000
100000
50000
0
Time ->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Retention Time

Fig. 5a. HPLC chromatogram of extract from a one day old SMM treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.54
0.65
1.01
1.16
4.17
4.54
5.11
5.75
6.34
6.93
7.49; 7.87
8.58
9.12
10.05
11.88
13.10
14.45
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 5b. HPLC chromatogram of extract from a three day old SMM treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.57; 0.64
0.98
1.21
2.33
2.81
4.44
7.72
8.31
8.87
9.76
10.44
11.44
12.54
0.11
1.25
2.38
3.52
4.65
5.78
6.92
8.05
9.19
10.32
11.45
12.59
13.72
14.85
15.99
17.12
18.26
19.39
Retention Time

Fig. 5c. HPLC chromatogram of extract from a five day old SMM treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
4.87
5.84
8.38
8.68
9.00
9.54
10.58
12.47
2.97
3.65
4.34
5.03
5.71
6.40
7.08
7.77
8.46
9.14
9.83
10.52
11.20
11.89
12.58
13.26
13.95
14.64
Retention Time

concentration. The numbers corresponding to the actual peak areas obtained from the HPLC chromatograms for all cultures tested are contained in Table 2.

Once it was determined that the SMM degraded chlordane, which microbial species was responsible for the degradation was then investigated. The chromatograms in figures 6a, 6b, 6c, 7a, 7b, 7c, and 8a, 8b, 8c illustrate the activities of the ensiling bacteria, *Chromatia spp.* and *Oscillatoria spp.*, respectively. In all cases, there was degradation, but the most noticeable was that of the *Chromatia spp.* If an SMM culture system is treated with high levels of chlordane, > 1,500 mg/l, and sealed so as to maintain ambient conditions, and if the light is subdued, about 10 lux, but the temperature kept constant at 28 °C, *Chromatia spp.* blooms rapidly under the *Oscillatoria spp.*(Fig. 9).

Figure 10a displays a comparison of the four systems in which degradation was observed while Figure 10b displays them individually against the control. In a stastical comparison of the degradation rates observed in the these four systems, an analysis of variance test was performed. This test revealed no significant difference in the degradation rates among the ensiling bacteria, *Oscillatoria spp.* and the SMM. The *Chromatia spp.*, however, had a significantly higher mean rate of degradation than the others.

Table 2. Peak areas obtained from HPLC analysis and the calculated avarage percent chlordane remaining (recovered) in the system.

SMM experiment

Day	Sample 1.	Sample 2.	Sample 3.	Control	$\bar{X}$ % recovery
1	12.6	11.5	9.0	12.5	88.2 ± 14
2	12.1	8.3	6.1	12.3	71.8 ± 24
3	9.5	5.2	6.0	11.6	59.4 ± 20
4	8.8	5.0	3.4	12.3	46.6 ± 23
5	6.5	3.7	2.8	11.9	36.4 ± 16

Oscillatoria spp. experiment

Day	Sample 1.	Sample 2.	Sample 3.	Control	$\bar{X}$ % recovery
1	10.4	7.5	10.8	12.2	78.4 ± 15
2	10.2	4.8	12.0	12.1	74.3 ± 31
3	5.4	8.0	6.0	12.2	53.6 ± 11
4	4.0	6.9	5.0	12.5	42.4 ± 12
5	4.1	5.2	4.8	12.0	39.2 ± 4

TABLE 2 (cont.)

Chromatia spp. experiment

Day	Sample 1.	Sample 2.	Sample 3.	Control	$\bar{X}$ % recovery
1	8.5	6.1	10.8	12.4	68.2 ± 19
2	11.1	4.1	3.1	12.5	48.8 ± 37
3	4.8	4.5	6.0	12.5	40.8 ± 6
4	6.4	2.5	5.4	11.9	40.0 ± 16
5	2.1	0.9	1.8	12.4	12.9 ± 5

Ensiling bacteria experiment

Day	Sample 1.	Sample 2.	Sample 3.	Control	$\bar{X}$ % recovery
1	12.2	8.0	11.8	12.2	87.4 ± 19
2	9.2	8.9	8.8	11.5	78.0 ± 9
3	9.0	8.9	7.5	12.0	70.6 ± 10
4	8.3	7.6	7.2	11.9	64.7 ± 11
5	6.6	7.1	7.1	12.0	57.5 ± 8

Fig. 6a. HPLC chromatogram of extract from a one day old ensiling bacteria treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.61; 0.75
4.64
5.81
7.53
8.00
8.31
8.62
8.96
9.48
10.53
11.30
12.40
0.11
1.25
2.39
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 6b. HPLC chromatogram of extract from a three day old ensiling bacteria treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.62: 0.75
1.21
1.43
2.88
4.85
5.85
7.57
8.37
8.65
9.02
9.58
10.60
11.44
12.51
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 6c. HPLC chromatogram of extract from a five day old ensiling bacteria treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.62
1.45
2.84
4.73
8.24
8.55
8.87
9.43
10.48
11.30
12.36
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 7a. HPLC chromatogram of extract from a one day old *Chromatia spp.* treatment culture.

Absorbance
Retention Time
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
0.57; 0.68; 0.75
2.81
3.20
3.83
4.12
4.76
5.62
5.85
5.98
7.31
7.53
8.11
8.35
8.67
9.08
9.66
10.61
11.48
12.50
13.64
17.02

Fig. 7b. HPLC chromatogram of extract from a three day old *Chromatia spp.* treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.65; 0.82
1.14; 1.29
1.41; 1.54
1.71; 1.91
2.45
2.90
3.47
3.71
4.55
5.29
5.75
6.05
6.95
8.40
8.73
9.05
10.63
12.57
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 7c. HPLC chromatogram of extract from a five day old *Chromatia spp*. treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0320
0.0400
0.67
1.06
1.63
1.99
2.48
4.42
0.11
1.25
2.38
3.52
4.65
5.78
6.92
8.05
9.19
10.32
11.45
12.59
13.72
14.85
15.99
17.12
18.26
19.39
Retention Time

Fig. 8a. HPLC chromatogram of extract from a one day old *Oscillatoria spp.* treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.58
1.10
1.23
1.40
2.29
2.51
3.01
3.54
3.82
4.14
4.76
4.84
5.30
5.88
6.01
6.77
7.04
7.36
7.59
8.08
8.43
8.72
9.15
9.74
10.80
11.38
12.07
12.70
13.91
15.53
17.31
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 8b. HPLC chromatogram of extract from a three day old *Oscillatoria spp.* treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.60; 0.71; 0.77
1.27
1.42
2.60
3.06
4.00
4.38
5.09
6.22
6.50
7.26
7.95
8.53
8.85
9.17
9.60
10.25
11.34
13.39
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 8c. HPLC chromatogram of extract from a five day old *Oscillatoria spp.* treatment culture.

Absorbance
0.0000
0.0080
0.0160
0.0240
0.0320
0.0400
0.62
0.73
2.52
6.10
7.34
8.76
9.07
9.39
9.92
11.07
13.11
0.11
1.25
2.38
3.51
4.65
5.78
6.91
8.05
9.18
10.31
11.45
12.58
13.71
14.85
15.98
17.11
18.25
19.38
Retention Time

Fig. 9. *Chromatia spp.* (red) colonizes and blooms under *Oscillatoria spp.* after treatment with high levels of chlordane.

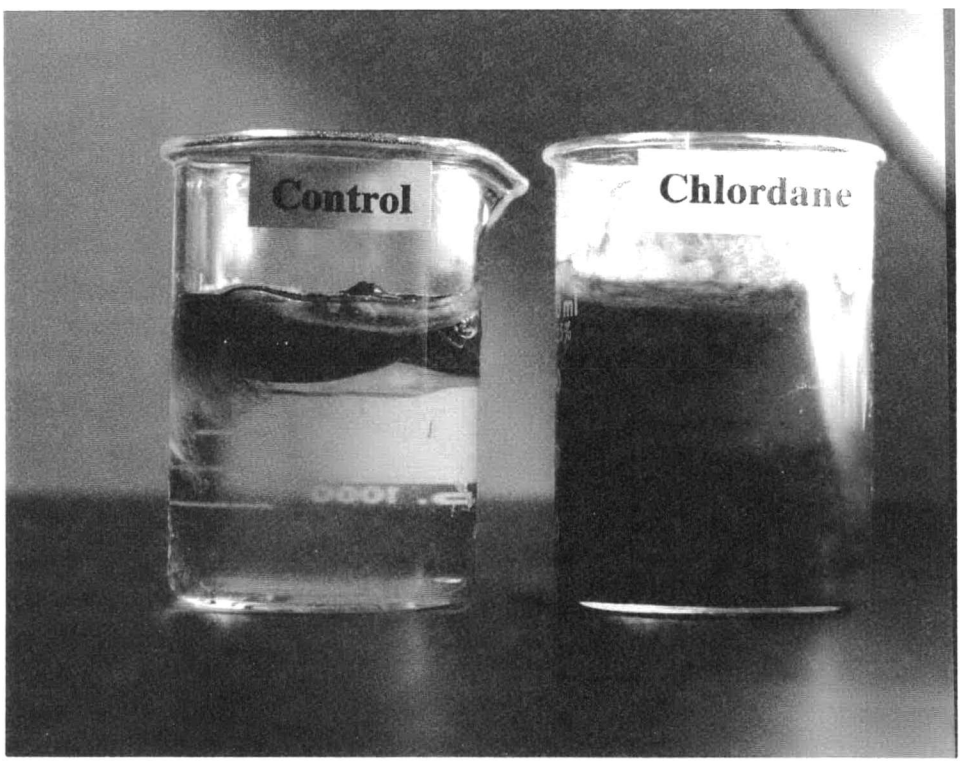

Control
Chlordane

Fig. 10a. Chlordane disappearance with time. A comparison of the degradation rates observed among SMM, ensiling bacteria, *Chromatia spp.* and *Oscillatoria spp.* Each point represents a mean value where n = 3.

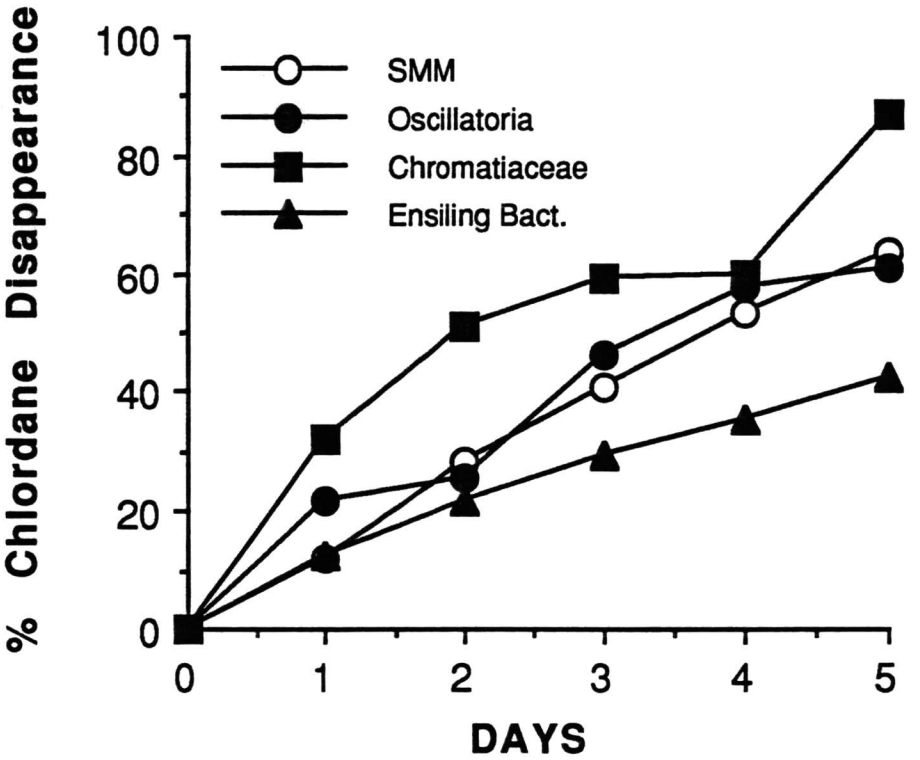
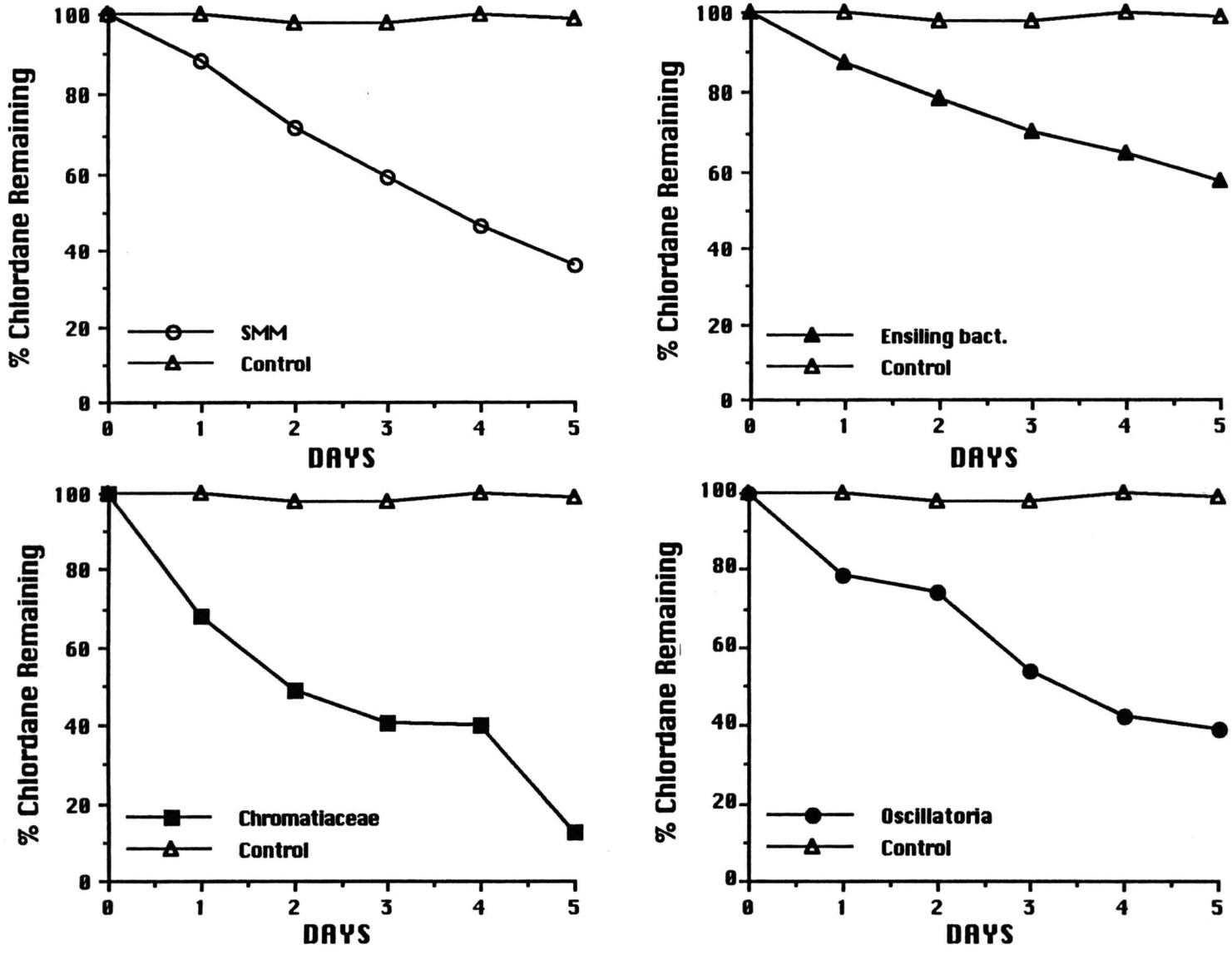

% Chlordane Disappearance
100
80
60
40
20
0
0
1
2
3
4
5
DAYS
SMM
Oscillatoria
Chromatiaceae
Ensiling Bact.

Fig. 10b. A plot of the degradation rates obtained in each system against a control system. Each point on the graph represents a mean value where n = 3.


% Chlordane Remaining
100
80
60
40
20
0
0
1
2
3
4
5
DAYS
SMM
Control
Ensiling bact.
Control
Chromatiaceae
Control
Oscillatoria
Control

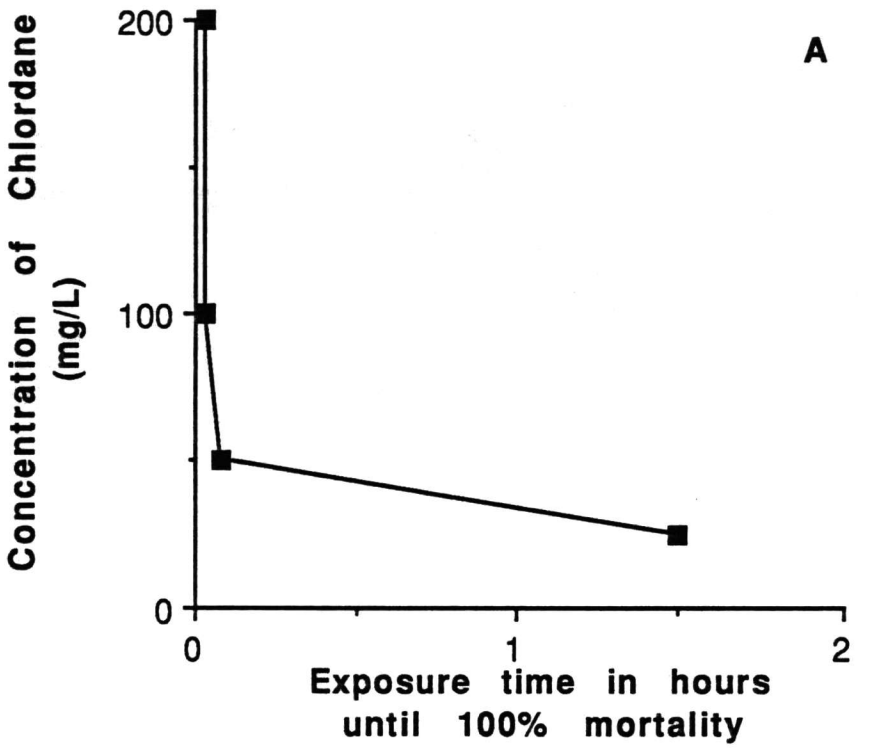
The area percent report obtained from the GC/MS for day five of the four systems in which degradation was observed, is displayed in Table 3. These results were in agreement with the results obtained from HPLC analysis. No degradation was observed with ensiled grass or the growth media, hence no chromatogram is shown for either of these.

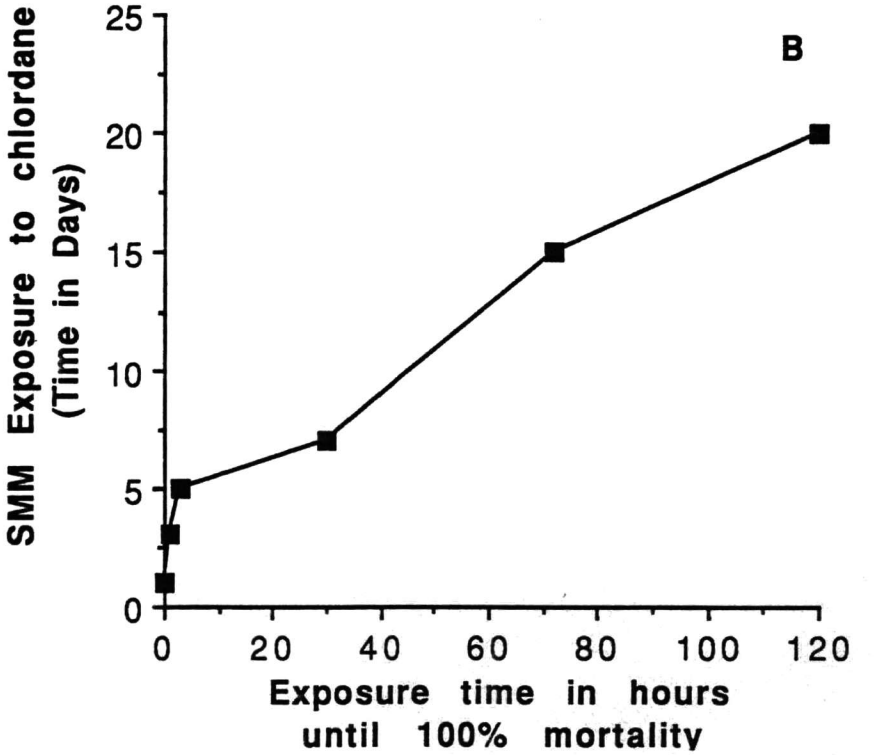
When copepods were exposed to 200 mg/l of chlordane, all the organisms died within two minutes. The mortality rate decreasd with decreasing levels of chlordane. When copepods were exposed to 50 mg/l of chlordane, all the organisms were disoriented at five minutes. They laid on their sides and made only circular movements. Five minutes later 50% of these organisms were dead. Figure 11 is a plot of the time taken to achieve 100% mortality against the concentration of chlordane in a treatment system.

Table 3. GC/MS area percent report. The values correspond to the percent chordane recovered from the individual culture after five days.

Culture type	% Chlordane recovered
SMM	40
Ensiling bacteria	51
Chromatia	14
Oscillatoria	41

Fig. 11. Copepod survival rate. A plot of the length of time copepods survived in various concentration of chlordane (A).
A plot of the length of time copepods survived in chlordane treated mat system of varying ages (B).


A
Concentration of Chlordane (mg/L)
200
100
0
0
1
2
Exposure time in hours until 100% mortality


B
SMM Exposure to chlordane (Time in Days)
25
20
15
10
5
0
0
20
40
60
80
100
120
Exposure time in hours until 100% mortality

CHAPTER V

DISCUSSION

This work did not attempt to determine the mechanism of degradation but rather to show the capability of the SMM to biodegrade chlordane and to identify the individual species within the SMM capable of degradation. Earlier attempts to biodegrade the hexachlorocyclopentadiene rings of chlordane and heptachlor (Kennedy et al., 1990) demonstrated that chlordane is more easily degraded than heptachlor. Based on the structural differences between the two compounds, it was assumed that the absence of a double bond between C-2 and C-3 of the chlordane molecule made that point vulnerable to the fungal enzymatic attack.

It is now known that no single species has a monopoly on the degradation process, but rather, like the established order within the SMM, the process is cooperative. Single species can, however, degrade the compound, but they seem to lose their degradative ability much sooner than does the whole mat. Figure 10b shows an almost straight line for the degradation process as observed in the SMM. Compare this with the leveling off periods observed in single speciea after day two or three (also shown in Fig. 10b). In addition, there are visible changes in growth patterns observed in single species. At day two of the experiment,

the ensiling bacteria seem to fall out of the media, depositing at the bottom of the beaker, leaving an almost clear water column above. The *Chromatia spp.* seem to follow a similar pattern but at a much reduced rate. While clearing of the water column is a desirable step in some remediation processes, high mortality rate among the organisms performing the degradation task is undesirable. *Flavobacterium* immobilized in polyurethane actively degraded PCP for up to 100 days (Crawford and O'Reilly, 1988). The *Oscillatoria spp.* did not fall out of the media but they appeared blanched at five days. In contrast, the SMM showed no signs of death but rather exhibited luxuriant growth. This early loss of degradative ability by single species might also be attributed to a build up of waste products due to their initial population increase and the accompanying loss of nutritional source. It should be noted that, apart from the Allen and Arnon media (supplied initially to all cultures), and the ensiled grass clippings (supplied to the *Oscillatoria spp.* and SMM during the pre-incubation period), no additional source of nutrition was provided. However, methanotrophic and heterotrophic bacterial bioreactors designed to biodegrade TCE, must be fed periodically with methane or propane as foodstock (Phelps, 1990). The symbiotic self-sufficient nature of the SMM is therefore a distinct asset for continuous growth and active degradation.

Kennedy et al., (1990) used a single species (*Phanerochaete chrysosporium*) and a much smaller population and media volume than was used here. Correspondingly, they reported lower rates of degradation, nanomoles in a thirty day treatment period, compared to millimolar levels in a five day period as reported here. Thus, a major difference between this works and that of Kennedy et al.(1990) is the quantity of the microbes added and the rates of degradation. *Phanerochaete chrysosporium* produces its degradative enzyme under nutritionally limiting conditions. The SMM, however, actively entraps the compound under normal growing conditions and proceeds with its degradation under nutrient self-supplying processes of photosynthesis and nitrogen fixation. This active sequestering of toxic materials, such as metals, from an aqueous media and from sediment surfaces by SMM has been demonstrated by this work and that of Ibeanusi (1989). This sequestering ability brings the compound into direct contact with a mass of chemically active cells thereby increasing the rate of degradation. Ritchie (1992) has shown that the cell wall of cyanobacteria behaves as a cation exchange material and binds cations. Furthermore the species *Synechococcus R-2* actively accumulates Cl^- in light and dark at pH 7.5 and a chlorine concentration of 0.508 mol/m^3. These conditions are very close to those that existed within the SMM and *Oscillatoria spp.* mats during the first three days after chlordane

treatment. It is possible therefore, that the degradative process might have followed the path of reductive dechlorination. Vogel and McCarty (1985) and Phelps et al.(1990) have suggested that TCE degradation in bioreactors occurred via reductive dechlorination in anaerobic microniche (microzone) although the bulk phase liquid remained oxidized. In the SMM the active photosynthetic activity of cyanobacteria ensured consistently high levels of oxygen in the system but the anaerobic zones persisted (Bender et al., 1989) (See Fig. 1). While the ensiling bacteria showed significant decrease in the rate of degradation at five days, there was no significant difference between the rates of degradation of the SMM, and *Oscillatoria spp.* over the same period ($p < .01$). However, it should be noted that the SMM is considered a superior system because of its self-sustaining abilities in terms of cell viability and nutrient production. Also, the multi-species character of the mat adds a durability that is unavailable in single species systems. In the preliminary stages of this work, SMM has shown the capability of dealing with levels of chlordane above 2,000 mg/l. SMM is viable for very long periods of time under adverse conditions with little or no maintenance. It can withstand periods of droughts while flood waters may serve as a vehicle of dispersal. The SMM is thus an excellent candidate for biodegradation of chlordane in a field application system.

It might be argued that the SMM simply entraps the chlordane in the mucopolysaccharide layer surrounding the cyanobacterial cell and hence bioaccumulates rather than biodegrades the compound. Besides the effective extraction procedure used, the bioassay defies and confounds this argument. When copepods were introduced into a SMM culture system one day after treatment with 200 mg/l of chlordane, all of the organisms died within five minutes. Copepods introduced into a similar system five days later, lasted eighteen minutes. When a three-week old culture system was used the organisms actively grazed on the SMM and survived beyond five days (See Fig. 11). Since copepods did not survive beyond 1.5 hours in the presence of 25 mg/l of chlordane, these data seem to suggest that a > 75% disappearance (probably about 90%) had occurred over the twenty day period.

The most rapid degradation rate was observed with the *Chromatia spp*. There was a significant difference ($p < .01$) between this and all other systems studied. One interesting observation is that *Chromatia spp*. population explodes in the presence of chlordane. This rapid increase in population could account for the rapid rate of degradation. However, this population increase becomes counter productive with time, as it leads to greater competition for nutrients in the absence of either medium supplements or the nutrient support of the mat; cell death occurs after two days. If

the *Chromatia spp.* is cultured in an SMM, however, its high population density is sustained. This sustained *Chromatia spp.* population density may be the result of an abundance of nutrient or growth factors produced by some other microbial species in response to chlordane treatment, or it may be due to loss of some competitive species due to chlordane poisoning.

While the ensiling bacteria had a high initial rate of degradation, this rate leveled out rapidly to the point where degradation was almost undetectable after six days (data not shown).

If the degradation rates of the various systems are added, it becomes obvious that the sum of the parts is greater than the whole. This makes sense only if the dynamics of the ecosystem are understood. At no point in the development of the SMM is the population density of any one organism near the population density observed in the single species experiment. This is because the ecosystem has a limit on its carrying capacity for any one species, if all species within the system are to occupy their proper niche. To allow any one specie to reach the numbers observed in the single species experiment compromises the natural integrity of the mat.

Further, there is no consistent degradation rate even among the individual species. This has its base in the growth and survival rate of the organisms. In some

cultures, the organisms remained in the lag phase much longer than those in other cultures, even though they all had the same growth conditions. This is responsible for the large standard deviation seen in TABLE 2. Those cultures that had long lag phases demonstrated slower degradation rates initially and were likely to remain in an active degradative state much longer. Organisms with long lag phases might be undergoing further acclimation and hence a delay in their ability to deal with the compound.

At first glance, single species might appear to be better candidates for biodegradation of chlordane. However, keeping dense, viable populations of single species for any extended period of time can pose a problem. Single species are unable to recycle their own waste and often fall victims of their own toxins. In the SMM, there is a symbiotic relationship. Cyanobacteria converts carbon dioxide to carbohydrates, fix nitrogen for protein synthesis and provides oxygen for aerobic respiration. Some anaerobes in the mat may also be nitrogen fixers, vitamin producers or just bioconverters of toxic intermediates into useful products. As a group, the organisms can carry out the biodegradation of chlordane for extended periods but as single species they poison themselves with their own waste. This is consistent with the reports of Little et al. (1988), Nelson et al. (1987) and Wackette and Gibson (1988) which claimed that complex microbial consortia may have a greater

ability to simultaneously degrade mixtures of organic waste than pure cultures. However, the addition of pure cultures to a consortium may further enhance its ability to biodegrade organic compounds.

Although chlordane was the featured compound in this work, based on the research reported by Kennedy et al. (1988) on the degradation of other structurally similar chlorinated pesticides by *Phanerochaete chrysisporium* (aldrin, dieldrin, heptachlor, lindane and mirex), it is reasonable to assume that the SMM may be able to effectively degrade these compounds as well. Further research is planned in this area. If this is the case, the SMM may represent an important economical bioremediation technology for contaminated soils. Bioremediation of chlorinated pesticides will help to improve the quality of our food, water, and air, which in turn improves the quality of life for everyone.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Over the last decade, we have seen landfill sites overflow with industrial and domestic waste, water bodies become lifeless due to toxic effluents and spills, food supplies contaminated with pesticides, the ozone layer depleting (supposedly from chloroflurocarbons, CFC's), and an increase in the incident of some forms of cancer. The need for remediation technologies is thus a reality. Remediation technologies are often costly and may create novel problems in terms of toxic degradation products. Microbial biodegradation, if economical and safe, is thus a real and viable option in remediation of soil and water. Several microorganisms have the capability to degrade specific chlorinated and aromatic compounds (Phelps et al., 1990). In such cases the compounds are mineralized to harmless products like carbon dioxide and water or co-metabolized and eventually integrated into the cell structure of the organism. Such remediation is environmentally sound and promotes a healthy human environment.

In this work, evidence for the biodegradation of chlordane was presented. Because cyanobacterial mats are ancient biological systems and occur naturally in all

environments, the system represents a safe choice for bioremediation of contaminated soils and water bodies. No special precautions are necessary since species are ubiquitous and there are no reported negative effects caused by *Oscillatoria spp.* under normal environmental conditions. The system presented here can serve as a model from which modified versions can be developed to meet a particular need.

It is obvious that many questions still remain to be answered, such as: What is the fate of the chlorine released from chlordane degradation? What are the structural identities of the by-products or intermediates? What is the mechanism of degradation? How can mats be safely acclimated and immobilized in large enough volumes for use in lakes and fields? Some of these questions are currently receiving attention as this work progresses.

BIBLIOGRAPHY

Agril, J. Thermal degradation of selected chlorinated herbicides. Food Chemistry 24(6):1194-1198; 1976.

American Chemical Society. Fate of organic pesticides in the environment. American Chemical Society 161st meeting 1972.

Anderson, A.C. Environmental toxicology-biodegradation of xenobiotics. Journal of Environmental Health 48(4): 196-199; 1990.

Allen, M.B. and Arnon, D.I. Studies on nitrogen-fixing blue-green algae 1. Growth and nitrogen fixation by *Anabaena cylindrica*. Lemm. Tl. Physiol. 30:366-372; 1955.

Barnes, R.D. Invertebrate zoology. 4th ed. Pennsylvania: Saunders College Philadelphia; 1980.

Barth, R. Chlorinated hydrocarbons. Microbial Ecology 12: 155-172; 1986.

Baxter, D. Chlordane: A pesticide review. National Coalation Against the Misuse of Pesticide 3-15; 1988.

Becker, S.S. and Sell, S. Cancer Research 39:3491-3494; 1979.

Bender, J., Gould, J.P., Vatcharapijarn, Y., Saha, G. Uptake, transformation and fixation of Se(VI) by a mixed selenium tolerant ecosystem. Water, Air, and Soil pollution 59:359-367; 1991.

Bender, J., Archibold, E.R., Ibeanusi, V., Gould, J. Lead removal from contaminated water by a mixed microbial system. Water Science and Technology 21(12):1661-1665; 1989.

Black, I.F. and Koeman, J.H. Future hazard from pesticide use. International Union for Conservation of Natural Resources, Commission on Ecology, paper No.4:100 pp; 1984.

Bumpus, J.A. Biodegradation of polycyclic aromatic hydrocarbons by Phanerochate chrysosporium. Applied and Environmental Microbiology 55(1):154-158; 1988.

Camara de Sanidad Agropecuaria y Fertilizantes: Guia de Productos Fitosanitarios para la Republica Argentina: 414 and 472-474; 1988.

Caumette, P. Ecology and general physiology of anoxygenic phototrophic bacteria in benthic environment. Cohen, Y. and Rosenberg, E. editors. Microbial mats. American Society for Microbiology, Washington, DC. 1989.

Cavalier, T.C., Lavy, T.L., Mattice, J.D. Degradation of chlorinated herbicide. Biological Abstracts 91(10):112457; 1991.

Crawford, R.L. and O'Reilly, K.T. Development of processes for biodegradation of pentachlorophenol. European Conference on Biotechnology 23:155-161; 1988.

Curley, A. and Garrettson, L.K. Toxic effects of selected pesticides. Archives of Environmental Health 18:211-215; 1969.

EPA. Office of General Counsel. FIFRA Docket 384:77-85; 1975.

EPA. Guidelines for the re-registration of pesticide products containing heptachlor as the active ingredient. Office of Pesticide Programs. Washington, DC 30-41; 1986a.

EPA. Carcinogenicity assessment of chlordane and heptachlor/heptachlor epoxide. Office of Health and Environmental Assessment. Washington, DC 1986b.

EPA. Recognition and management of pesticide poisoning. 19; 1989.

Epstein, S.S. and Ozonoff, D. Leukemias and blood dyscrasias following exposure to chlordane and heptachlor. Carcinogenesis, Mutagenesis and Teratogenesis 7:527-540; 1987.

Fliermans, C.B. Phelps, T.J. Ringelberg, D. Mikell, A.T. White, C.D. Mineralization of TCE by heterotrophic enrichment cultures. Applied Environmental Microbiology 54:1709-1714; 1988.

Fogg, G.E., Stewart, W.D.P., Fay, P., Walsby, A.E. The blue-green algae. Academic Press, New York. 1973.

Ford, W.M. and Hill, P.E. Organochlorine pesticides in soil sediments and aquatic animals in the Upper Steel Bayou

watershed of the Mississippi. Archives of Environmental Contamination and Toxicology 20:161-167; 1991.

Georgia Department of Natural Resources. Fishermen advisory. 1991.

Gosselin, R.E. Clinical toxicology of commercial products. 4th ed. Williams and Wilkins Co. Baltimore, Maryland; 1976.

Greenpeace Report. Exporting banned pesticides: Fueling the circle of poison. 2-25; August 1989.

Hansen, G.W.; Oliver, F.E.; Otto, N.E. Herbicide manual. A water resource technical publication. U.S. Bureau of the Interior, Denver, Colorado: 256-260; 1983.

Hearing Records, H.R. 2622. A bill to amend the Clean Air Act to regulate the emission of certain hazardous air pollutants. Subcommittee on Health and Environment of the Committee on Energy and Commerce. Serial No. 100-8,24 pp; 1987.

Henry, R.A., Schmit, J.A., Dheckman, J.F. Analytical chemistry. New York: John Wiley & Sons; 1971.

Ibeanusi, V. Mechanisms of heavy metal uptake in a mixed microbial system. (Dissertation); 1989.

Kearney, P.C. and Kufman, D.D. Herbicide chemistry, degradation and mode of action: U.S Bureau of the Interior; 1988. 2 vol.

Kennedy, D.W., Aust, S.D., and Bumpus, J.A. Comparative biodegradation of alkyl halide insecticide by the white rot fungus *Phanerochaete chrysosporium* (BKM-F-1767). Applied and Environmental Biology. 56(8):2347-2353:1990.

Kerby, N.W. and Stewart, W.D.P. Biotechnology of microalgae and cyanobacteria. Biochemistry of the algae and cyanobacteria. Oxford Science Publishers, New York, New York; 1988.

Kirkland, J.J., Holt, R.F., Pease, H.L. Determination of benomyl residues in soil and plant tissue by high speed cation exchange liquid chromatography. Journal of Agriculture & Food Chemistry 21:368; 1973.

Little, C.D., Palumbo, A.V., Herbes, S.E., Lindstrom, M.E., Tyndall, R.L., Gilmer, P.J. Trichloroethylene

biodegradation by a methane-oxidizing bacterium. Applied Environmental Microbiology 54:951-956; 1988.

Matsumura. Boush. Misato., editors. Environmental toxicology of pesticides. Academic Press, New York:1972.

Merck Manual. 10th ed. Merck Co. Rahway, New Jersey; 1983.

Mondecar, M., Bender, J., Ross, J., George, W., Dummons, S. Bioremediation of TNT by a mixed microbial ecosystem. (Abstract); American Society for Microbiology. General meeting, New Orlenes, LA.; 1992

Moye, H.A. Analysis of pesticide residue. Wylie, New York, New.York; 1980.

Muirhead-Thomas, R.C. Pesticide impact on stream fauna with special reference to macroinvertebrates. New York: Cambridge University Press; 1989.

Nelson, M.J., Montgomery, S.O., Mahaffey, W.R., Pritchard, P.H. Bioremediation of TCE and invilvement of an aromatic biodegradative pathway. Applied Environmental Microbiology 53:945-954; 1987.

New York State Department of Environmental Conservation. Draft environmental impact statement on amendments to 6 NYCRR part 326. Albany, New York. 1986: 100-123.

Osselton, M.D. and Snelling, D.R. Chromatographic identification of pesticides. Journal of Chromatography 306:256-271; 1986.

Parlar, H. Organochlorides. Journal of Agriculture and Food Chemistry. 27:278-283; 1979.

Paerl, H.W. Interaction with bacteria. Carr, N.G. and Whitton, B.A., editors. The biology of cyanobacteria. California: University of California Press, Berkeley. 441-459; 1982.

Paerl, J.H. and Kellar, P.E. Nitrogen-fixing *Anabaena*: Physiological adaptation instrumental in maintaining surface bloom. Science 204:620-622; 1979.

Phelps, T.J., Niedzielski, J.J., Schram, R.M., Herbs, S.E., White, D.C. Biodegradation of TCE in continuous-

recycle expanded-bed bioreactors. Applied Environmental Microbiology 56:1702-1709; 1990.

Phillips, P., Bender, J., Word, J., Niyogi, D., Denovan, B., MS in preparation. Biodegradation of naphthalene, phenanthrene, chrysene and hexadecane with mixed microbial mat.

Pimentel, D., McLaughlin, L., Zepp, A., Latikan, B., Kraus, T., Kleinman, P., Vancini, F., Roach, J.W., Graap, E., Keeton, S.W., and Selig, G. Environmental and economic effects of reducing pesticide use. Bioscience 41(6):402-409; 1991.

Ritchie, R.J. The cyanobacterium *Synechococcus* R-2 (*Anacystis nidulans, S. lepoliensis*) PCC 7942 has a sodium-dependent chloride transporter. Plant, Cell and Environment 15:163-177; 1992.

Rochkind, M.L. Microbial decomposition of chlorinated aromatic compounds. U.S. EPA, Cincinnati, Ohio. EPA/600/2-98/090; 1989.

Rudd, R.L. Pesticide and the living landscape. The University of Wisconsin Press, Madisen, Wisconsin; 1966.

Sale, D.R. Bacterial inoculants as silage additives. Journal of Applied Bacteriology, Symposium Supplement: 9s-26s; 1986.

Sherman, J. Chemical exposure and disease: Diagnostic and investigative techniques. Van Nostrand Reinhold; 1988.

Smith, A.J. Modes of cyanobacteria carbon metabolism. Carr, N.G. and Whitton, B.A., editors. The biology of cyanobacteria. California: University of California Press, Berkley. 47-84; 1982.

Stacey, C.I. and Tatum, T. House treatment with organochlorine pesticides and their levels in human milk. Bulletin of Environmental Contamination and Toxicity. 35:202-209; 1985.

Steiert, J.G., Pignatello, J.J., Crawford, R.L. Degradation of chlorinated ppphenol by a pentachlorophenol-degrading bacterium. Applied and Environmental Microbiology 53(5): 907-910; 1987.

St. Vincent and the Grenadines Agriculture Report. Use of agrochemicals in St. Vincent and the Grenadines. 16 pp; 1989.

The Vincentian News. Pesticide horror in the banana industry. Friday June 1, 1990.

U.S. Department of Energy. Evolution of mid-to-late basic research for environmental restoration . Washington D.C. DOE/ER-0419: 23-25; 1989.

U.S. Department of Health, Education, and Welfare. Bioassay of chlordane for possible carcinogenicity. 1971.

Vogel, T.M. and McCartey, P.L. Microbial degradation of mixed organic waste. Applied Environmental Microbiology 49:1080-1083; 1985.

Wackett, L.P. and Gibson, D.T. Degradation of TCE by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Applied Environmental Microbiology 54:1703-1708; 1988.

Wilson, J.T. and Wilson, B.H. Methanotrophic bacteria. Applied Environmental Microbiology 49:242-243; 1985.

Weisskopf, M. The human toll from tainted milk: Farming families, nursing mothers, babies in the trail of heptachlor, Washington Post. 1986 March 31.

Wheeler, W.B and Thompson, N.P. Analysis of chlorinated hydrocarbons. Moye, H., editor. Analysis of pesticide residue. Wiley, New York. 199-218; 1980.